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PRINCIPAL INVESTIGATOR: Susan J. Knox, M.D., Ph.D.

CONTRACTING ORGANIZATION: Stanford University
Stanford, California 94305-5401

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Susan J. Knox, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Stanford University
Stanford, California 94305-5401

E-Mail: sknox@stanford.edu

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A large body of data suggests that selenium supplementation may be used as a chemopreventative strategy to reduce the risk of prostate cancer. In spite of this, little is known regarding the use of selenium as a cancer therapy. The inorganic form of selenium, selenite, undergoes thiol-dependent reduction to selenide which supplies selenium for the synthesis of selenoproteins. Excessive selenite metabolism depletes cells of the primary intracellular antioxidant, glutathione, and generates superoxide. The net effect of the metabolism of selenite is a profound alteration in the cellular redox status and generation of potentially lethal reactive oxygen species. We characterized the response of androgen-dependent LAPC-4 prostate cancer cells, as well as patientmatched pairs of normal and malignant prostate cells to selenite. Selenite-induced growth inhibition and apoptosis were correlated with changes in Bcl-2 family member expression, altered intracellular GSH status, and MnSOD expression. We also examined the ability of selenite to sensitize prostate cancer cells to ©-irradiation. The primary goal of this proposal was to generate preclinical data supporting the concept that selenite might be useful as a novel chemotherapeutic agent alone or in combination with radiation therapy to treat prostate cancer.

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Introduction

A large body of data suggests that selenium supplementation may be used as a chemopreventative strategy to reduce the risk of prostate cancer. In spite of this, little is known regarding the use of selenium as a cancer therapy. The inorganic form of selenium, selenite, undergoes thiol-dependent reduction to selenide which supplies selenium for the synthesis of selenoproteins (1). At lower concentrations, the major effects of selenite are related to its role as a micronutrient. However at higher concentrations, selenite may become toxic. Excessive selenite metabolism depletes cells of the primary intracellular antioxidant, glutathione, and generates superoxide (Figure 1) (2,3). The net effect of the metabolism of selenite is a profound alteration in the cellular redox status and generation of potentially lethal reactive oxygen species. We characterized the response of androgen-dependent LAPC-4 prostate cancer cells, as well as patient-matched pairs of normal and malignant prostate cells to selenite. Selenite-induced growth inhibition and apoptosis were correlated with changes in Bcl-2 family member expression, altered intracellular GSH status, and MnSOD expression. We also examined the ability of selenite to sensitize prostate cancer cells to γ-irradiation. The primary goal of this proposal was to generate preclinical data supporting the concept that selenite might be useful as a novel chemotherapeutic agent alone or in combination with radiation therapy to treat prostate cancer.

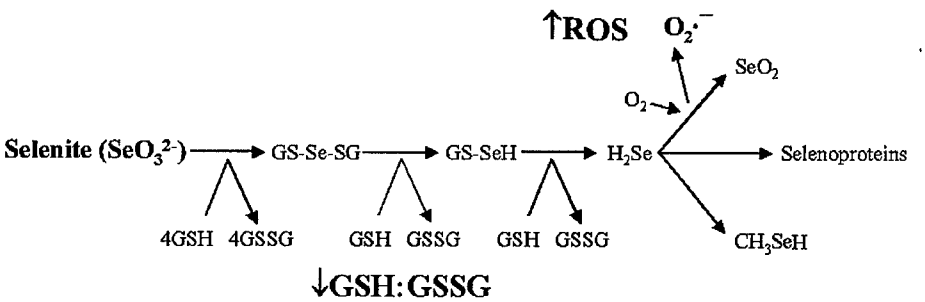

Fig. 1. Schematic illustration showing the metabolic reduction of selenite with GSH.

Body

Task 1. Study the effects of selenite on apoptosis and cell survival in LAPC-4 cells and primary prostate cancer cell strains *in vitro* and *in vivo*.

Selenite-induced growth inhibition and apoptosis in LAPC-4 prostate cancer cells

Selenite inhibited cell growth and induced apoptosis in a dose-dependent fashion in androgen-dependent LACP-4 human prostate cancer cells *in vitro*. The proliferation of LAPC-4 cells was measured using the MTS assay after incubation with selenite for 48 hours. Cell proliferation was 53.3% of control after treatment with 10 μM selenite, and 33.4% of control after treatment with 25 μM selenite (Figure 2A). Apoptosis was measured as the percentage of cells in the sub-G1 fraction of the cell cycle. The percentage of sub-G1 cells following treatment with 10 or 25 μM selenite for 48 hours was 14.5% and 26.1%, respectively (Figure 2B). Cleavage of caspase-3, a marker of apoptosis, was also detected in selenite-treated LAPC-4 cells by Western blotting (Figure 2C). In contrast, primary cultures of normal prostate epithelial cells were more resistant to selenite-induced apoptosis than LAPC-4 cells (Figure 2D). These results are consistent with other reports showing that selenite has differential effects in prostate cancer versus normal cells (4,5).

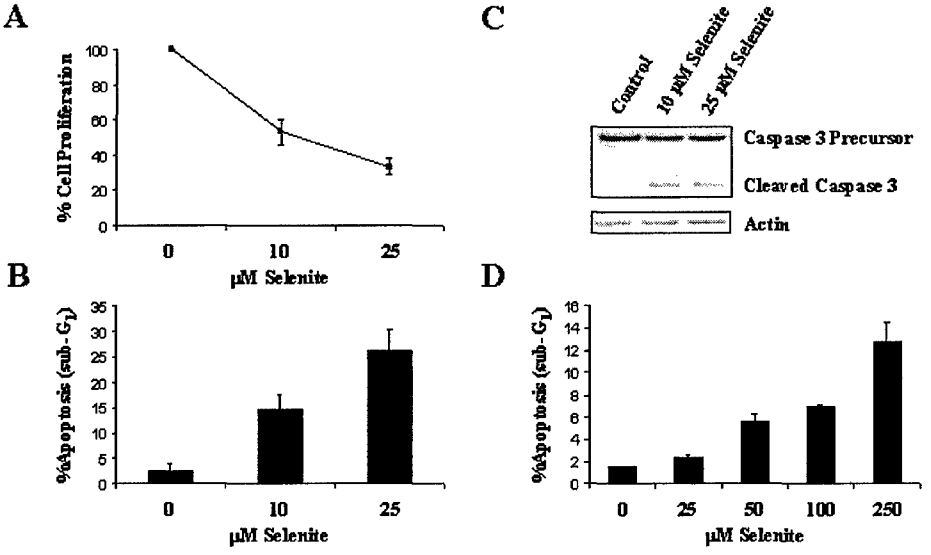

Fig. 2. Selenite-induced growth inhibition and apoptosis in LAPC-4 prostate cancer cells and normal prostate cells. *A*, LAPC-4 cells were treated with selenite at the indicated concentrations for 48 hours and cell proliferation was measured by MTS assay. *B*, LACP-4 cells were treated with selenite for 48 hours and percent apoptosis was detected as the fraction of cells in sub-G_1. *C*, Caspase-3 cleavage in LAPC-4 cells following exposure to selenite for 48 hours as detected by Western blot analysis. Actin protein expression was used to normalize for loading. *D*, Primary cultures of normal prostate cells treated with selenite for 48 hours and percent apoptosis was detected. Values represent the mean ± SD for 3 experiments.

Effects of selenite on intracellular GSH and GSSG in LAPC-4 cells

Since oxidative stress is induced by selenite, we measured changes in the intracellular redox state by measuring total GSH and GSSG concentrations at 6, 24, and 48 hours after treatment with 10 μM selenite. Selenite decreased total GSH levels in a time-dependent fashion from a basal level of 52.1 ± 5.6 nmol/mg to 11.8 ± 2.1 nmol/mg at 48 hours (Figure 3A). The concentration of GSSG increased following selenite treatment and was maximal after 24 hours (Figure 3B). As a result, the ratio of GSH:GSSG in LAPC-4 cells was decreased by selenite (Figure 3C). As early as 6 hours after treatment with 10 μM selenite, the GSH:GSSG ratio was decreased from 129.4 ± 13.6 to 15.1 ± 2.3. These changes in intracellular GSH content in response to selenite indicate a dramatic shift in the cellular redox balance towards an oxidative state.

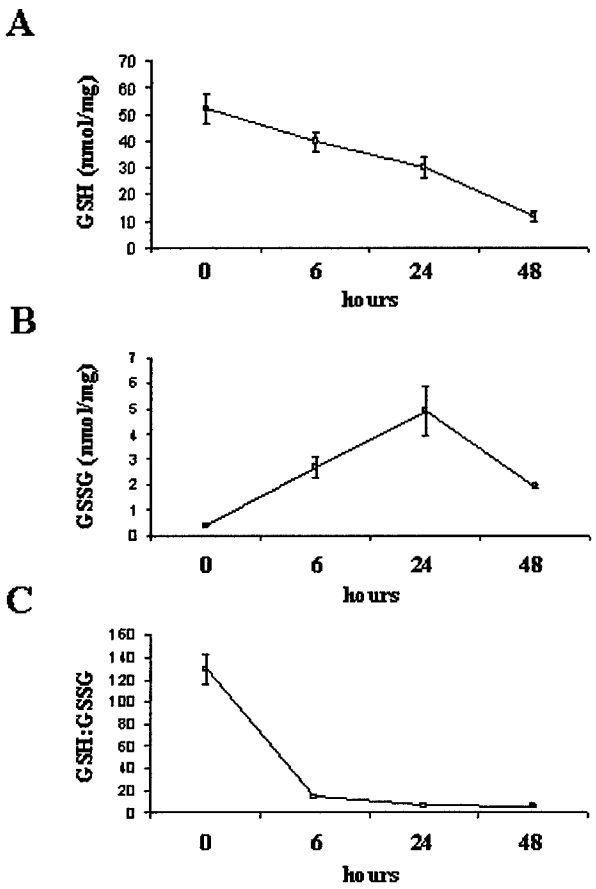

Fig. 3. Effects of selenite on intracellular GSH content in LAPC-4 cells. Cells were treated with 10 μM selenite for 6, 24, and 48 hours and total GSH and GSSG concentrations were measured using the GSH-reductase recycling assay. Changes in *A*, total GSH, *B*, GSSG, and *C*, GSH:GSSG are shown after treatment with selenite for 6-48 hours. Values represent the mean ± SD for 3 experiments.

Effects of selenite on bcl-2, bcl-xl, and bax expression in LAPC4 cells

Bcl-2, bcl-x_L, and bax are members of the Bcl-2 family that play key roles in the regulation of apoptosis (6). Therefore, we measured the expression levels of bcl-2, bcl-x_L, and bax after exposure to selenite for 48 hours by Western blotting (Figure 4). The expression of anti-apoptotic, bcl-2 and bcl-x_L, were decreased following treatment with selenite, and this reduction was coupled to an increased expression of pro-apoptotic bax. The decreased bcl-2:bax expression ratio suggests that selenite-induced apoptosis in LAPC-4 cells correlates with a shift in the balance of Bcl-2 family member expression from a pro-survival to apoptotic state.

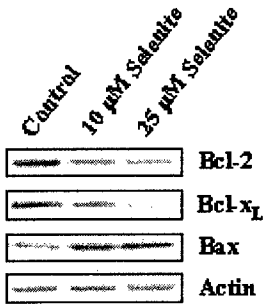

Fig. 4. Effects of selenite on bcl-2, bcl-x_L, and bax protein expression. Western blot analysis of bcl-2, bcl-x_L, and bax-α expression in LAPC-4 cells after treatment with selenite for 48 hours. Actin protein expression was used to normalize for loading.

Effecs of selenite on the growth of LAPC-4 xenograft tumors in nude mice

In vivo pilot studies were performed in male and female nude mice with subcutaneous LAPC-4 xenograft tumors. Female mice were used because under the selective pressure of androgen deprivation, LAPC-4 tumors reproducibly evolve to a hormone refractory state, thereby providing a model for the study of androgen-independence (7). Once tumors reached approximately 100 mm^3 in size the mice were treated with 2 mg/kg selenite three times per week. Selenite treated mice showed significant tumor growth inhibition compared to untreated control animals (Figure 5). In addition, there was no observed depression of body weight in selenite treated mice relative to control mice (data not shown). The results demonstrate that selenite can delay the growth of both androgen-dependent and androgen-independent LAPC-4 tumors and the systemic toxicity of selenite may not mitigate potential therapeutic efficacy.

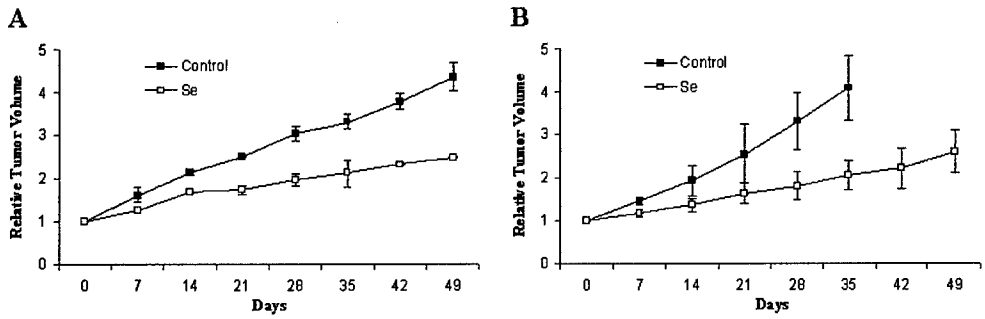

Fig. 5. Effects of selenite on the growth of LAPC-4 xenograft tumors in *A*, male and *B*, female mice. Mice were treated with 2 mg/kg selenite i.p. three times per week. Tumor volume was calculated weekly (tumor volume = π/6 x length x width x height).

Task 2. Show that primary prostate epithelial cells are more sensitive to selenite-induced apoptosis than normal cells and correlate these findings with differential expression of antioxidants (GSH and MnSOD) and Bcl-2 family members.

Selenite-induced apoptosis in normal and malignant prostate cells

Three patient-matched pairs of normal and cancer cell strains were available for study (PZ/CA-1, PZ/CA-2, and PZ/CA-3). Normal cell strains were obtained from a region of the peripheral zone (PZ) not involved in cancer. CA-1, CA-2, and CA-3 cell strains were derived from prostatic adenocarcinomas of Gleason grades 3/4, 4/3, and 4/3, respectively. A normal cell strain derived from the central zone (CZ) was also studied. In each case, the prostate cancer-derived cells, CA-1, CA-2, and CA-3, were more sensitive to selenite-induced apoptosis at 48 hours than the corresponding normal cells derived from the peripheral zone, PZ-1, PZ-2, and PZ-3, respectively (Figure 6). Normal cells derived from the central zone of the prostate were found to be as resistant to selenite-induced apoptosis as normal cells from the peripheral zone (Figure 6E). It should be noted that supra-physiological doses of selenite (25 - 100 μM) were required to induce apoptosis in the primary cell cultures at 48 hours, while LNCaP, LAPC-4, PC-3, and DU 145 prostate cancer cells have been shown to respond to much lower doses of selenite (≤ 10 μM) (4).

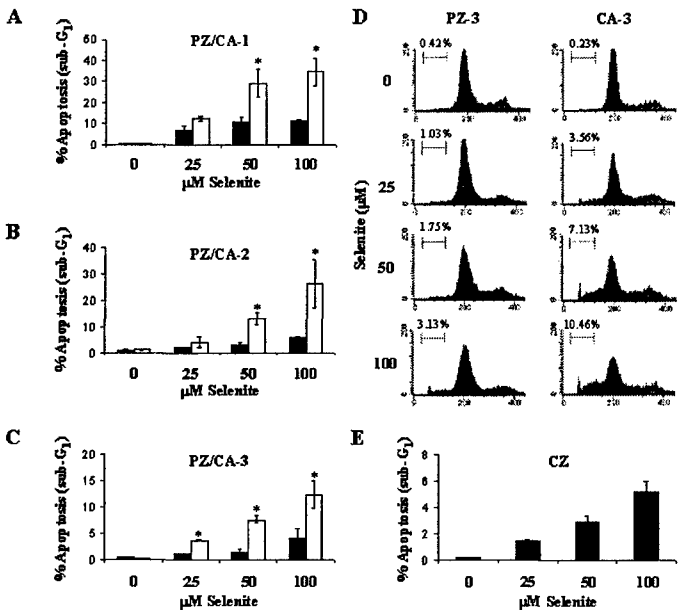

Fig. 6. Selenite-induced apoptosis in normal and cancer cell cultures. Cells were treated with selenite for 48 hours and apoptosis was detected as the fraction of cells in sub-G_1; normal prostate (PZ) (filled bars) and prostate cancer (CA) (open bars). *A*, *B*, and *C*, Effects of selenite on matched pairs PZ/CA-1, PZ/CA-2, and PZ/CA-3, respectively. *D*, Representative examples of cell cycle profiles of PZ/CA-3 cells and sub-G1 determinations following selenite treatment. *E*, Selenite-induced apoptosis in normal cells derived from the central zone (CZ). Values represent the mean ± SD for 3 individual experiments. * denotes a significant difference from normal cells ($p < 0.05$).

Intracellular GSH patient-matched normal and malignant prostate cells

GSH is considered to be a major component of selenite metabolism, and reactive oxygen species are produced as a result of the reaction of selenite with reduced GSH. Therefore, although GSH is the predominant intracellular antioxidant, it can also act as a pro-oxidant by facilitating selenite metabolism (8). Since the cytotoxicity of selenite is dependent upon its metabolism, we measured total GSH concentrations in normal and malignant prostate cells. Using the GSH-reductase recycling assay we found that total GSH concentrations were similar in normal and cancer cells derived from the same patient (Figure 7). In comparison, the concentrations of total GSH in PC-3, DU 145, and LNCaP prostate cancer cells were significantly lower than the primary cell cultures. Prostate cancer cell lines are extremely sensitive to selenite-induced apoptosis relative to primary cell cultures and this difference in GSH content may influence their response to selenite.

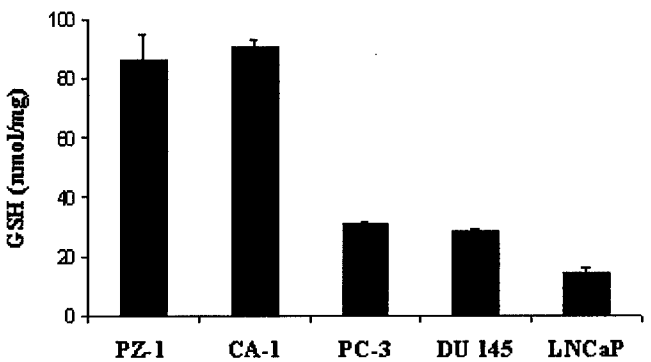

Fig. 7. GSH content in normal (PZ-1) and cancer (CA-1) cell cultures and in the prostate cancer cell lines, PC-3, DU 145, and LNCaP. Total GSH was measured using the GSH reductase recycling assay. Values represent the mean ± SD for 3 experiments.

Effects of selenite on bcl-2 and bax expression in normal and malignant prostate cells

We measured the expression levels of bcl-2 and bax after exposure to selenite for 48 by Western blotting (Figure 8). We noted that basal levels of bcl-2 were higher in cancer cells compared to normal cells in each matched pair, and we found that exposure to selenite for 48 hours decreased bcl-2 protein expression in all three prostate cancer-derived cell strains CA-1, CA-2, and CA-3. The expression of pro-apoptotic bax was induced in CA-1 and CA-2 prostate cancer cells and unchanged in CA-3 cells following treatment with selenite. In normal prostate cells the bcl-2:bax expression ratio was largely unaffected by selenite. PZ-1 and PZ-3 cells showed no change in bcl-2 or bax protein expression in response to selenite. In PZ-2 cells the expression of both bcl-2 and bax was increased following treatment with selenite. Therefore, the tumor-specific killing by selenite in prostate cancer cells appears to correlate with a decreased bcl-2:bax expression ratio.

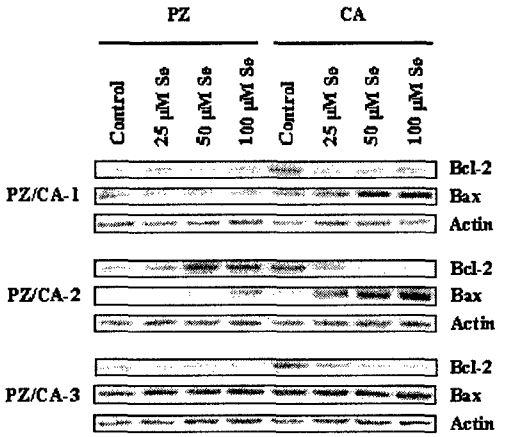

Fig. 8. Effects of selenite on bcl-2 and bax protein expression in normal and cancer cell cultures. Western blot analysis of bcl-2 and bax expression in PZ/CA-1, PZ/CA-2, and PZ/CA-3 cells following treatment with selenite for 48 hours. Actin expression was used to normalize for protein loading.

MnSOD expression in normal and malignant prostate cells

MnSOD is an anti-oxidant enzyme located in the mitochondrial matrix that scavenges superoxide anions. Since superoxide is a by-product of selenite metabolism, we measured MnSOD protein expression in the normal and cancer cell strains. Normal cells had higher basal levels of MnSOD protein expression than cancer cells (Figure 9A). The differences in MnSOD protein expression correlated with SOD activity in PZ-1 and CA-1 cells (Figure 9B). We also evaluated the expression of MnSOD in the tissues from which the cell strains (PZ/CA-2) were originally derived. MnSOD immunoreactivity was greater in normal regions of the prostate than in the areas containing cancer (Figures 9C). The results demonstrate the ability of primary cultures of normal and cancer-derived prostatic epithelial cells to represent important features of the tissues from which they were derived. Increased MnSOD protein expression in normal cells may contribute to their decreased sensitivity to selenite-induced apoptosis and suggest that MnSOD expression may be a predictive marker for the therapeutic response to selenite.

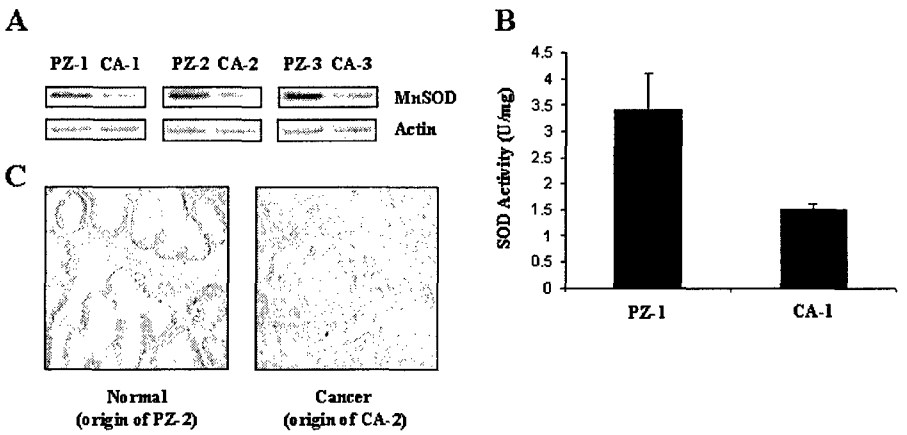

Fig. 9. MnSOD expression and activity in normal and cancer cell strains. *A*, Western blot analysis of MnSOD protein expression in PZ/CA-1, PZ/CA-2, and PZ/CA-3 cells. Actin protein expression was used to normalize for loading. *B*, SOD activity in PZ-1 and CA-1 cells. Values represent the mean ± SD for 3 experiments. *C*, Immunohistochemical staining of MnSOD in normal and cancerous epithelium of the prostate.

Effects of a MnSOD mimetic on cellular sensitivity to selenite

As shown previously, CA-1 cells are deficient in MnSOD relative to PZ-1 cells and more sensitive to selenite-induced apoptosis. Therefore, we tested the ability of a MnSOD mimetic, MnTMPyP, to protect CA-1 cells against selenite-induced cell killing. CA-1 cells were treated with or without selenite for 48 hours in the absence or presence of MnTMPyP (Figure 10). The results demonstrated that increasing MnSOD activity via the addition of MnTMPyp could protect CA-1 cells against selenite-induced apoptosis. The data also provides further evidence that selenite causes cell death via the production of superoxide radicals.

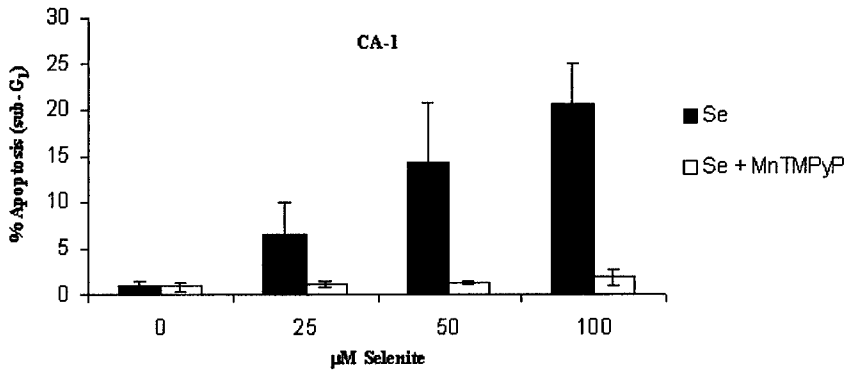

Fig. 10. MnTMPyP inhibits selenite-induced apoptosis in malignant prostate epithelial cells. CA-1 cells were treated with or without selenite for 48 hours in the absence or presence of 5 µM MnTMPyP and apoptosis was detected as the fraction of cells in sub-G_1. Values represent the mean ± SD for 3 individual experiments.

Task 3. Study the effect of combining selenite with radiation on apoptosis and overall tumor cell killing in primary prostate cancer cell strains and LAPC-4 cells *in vitro* and *in vivo*.

Selenite Sensitizes LAPC-4 Cells to Radiation-Induced Cell Killing

Since selenite-induced apoptosis in LAPC-4 cells correlated with decreased bcl-2:bax and GSH:GSSG ratios, we next tested the ability of selenite to sensitize LAPC-4 cells to γ-irradiation. LAPC-4 cells were treated with 10 µM selenite for 6 hours prior to receiving γ-irradiation, and survival was measured using a clonogenic asssay. This treatment regimen was based upon our data showing that treatment of LAPC-4 cells with 10 µM selenite for 6 hours decreased the GSH:GSSG ratio 88.3% (Figure 3). The surviving fraction of LAPC-4 cells after treatment with selenite alone was 0.431 ± 0.021 (data not shown). In experiments, in which

selenite was combined with radiation, the results were normalized for the killing from selenite alone. We found that selenite enhanced radiation-induced inhibition of colony formation (SF_2 = 0.056) compared to cells treated with radiation alone (SF_2 = 0.244) (Figure 11). These results indicate that selenite inhibits the clonal growth of LAPC-4 cells and enhances the effect of radiation on these cells.

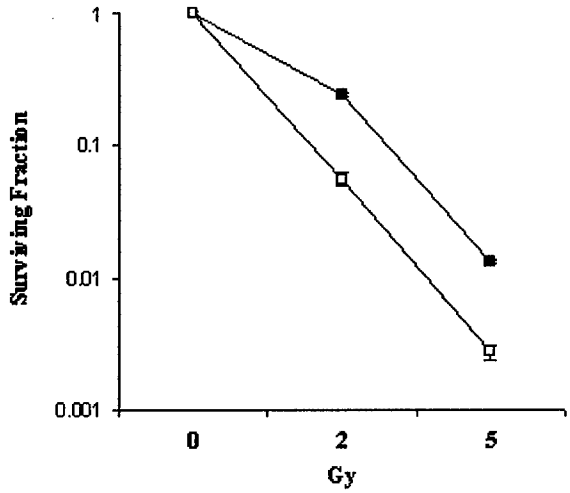

Fig. 11. Selenite enhances radiation-induced cell killing in LAPC-4 cells. Clonogenic survival data for LAPC-4 cells treated with radiation alone (■) or 10 μM selenite for 6 hours (□) prior to 2 or 5 Gy of γ-irradiation. Surviving fractions were calculated as the plating efficiency of treated cells divided by the plating efficiency of untreated cells. For combination experiments the results were normalized for the killing from selenite alone. Values represent the mean ± SD for 3 experiments.

Radiosensitization of androgen-independent DU 145 cells by selenite

We expanded our studies to include an androgen-independent prostate cancer cell line to determine if selenite would similarly sensitize these cells to γ-irradiation. The androgen-independent DU 145 prostate cancer cell line was chosen because previous studies have shown that selenite can inhibit growth and induce apoptosis in these cells (4). Again, changes in intracellular GSH and GSSG were measured 6 and 12 hours after treatment with 10 μM selenite. The ratio of GSH:GSSG decreased from a basal level of 146.4 ± 14.0 in controls cells to 57.5 ± 13.8 and 5.7 ± 1.8 at 6 and 12 hours, respectively (Figure 12A). Next, the effects of selenite on the response of DU 145 cells to γ-irradiation were studied using clonogenic survival assays. The surviving fractions of DU 145 cells treated with 10 μM selenite for 6 or 12 hours alone were 0.941 and 0.409, respectively (data not shown). After normalization for the killing from selenite alone, we found that pre-treatment with selenite enhanced radiation-induced cell death (SF_2 = 0.343 and 0.199 at 6 and 12 hours, respectively) compared to cells treated with radiation alone (SF_2 = 0.554) (Figure 12B). These results are summarized in Table 1.

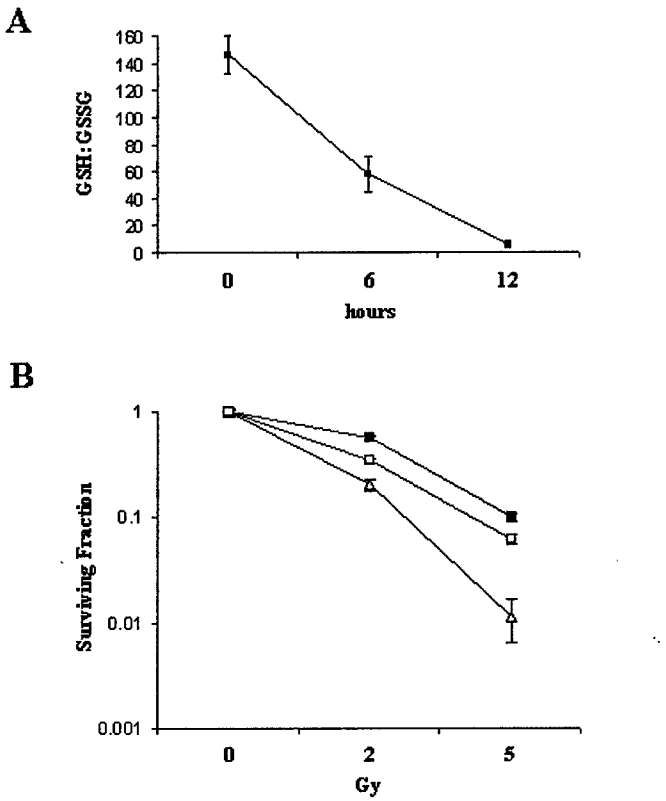

Fig. 12. Radiosensitization of DU 145 cells by selenite. A, Cells were treated with 10 μM selenite for 6 or 12 hours and changes in the ratio of GSH:GSSG were measured as described previously. B, Clonogenic survival data for DU 145 cells treated with radiation alone (■) or 10 μM selenite for 6 (□) or 12 (△) hours prior to γ-irradiation. Surviving fractions were calculated as the plating efficiency of treated cells divided by the plating efficiency of untreated cells. For combination experiments the results were normalized for the killing from selenite alone. Values represent the mean ± SD for 3 experiments.

Table 1. Radiosensitization of LAPC-4 and DU 145 cells by selenite

Cell Line	Treatment	SF_2	SF_2 ER
LAPC-4	Radiaton alone	0.244	
	10 μM Selenite 6 hr pre Radiation	0.056	4.36
DU 145	Radiaton alone	0.554	
	10 μM Selenite 6 hr pre Radiation	0.343	1.62
	10 μM Selenite 12 hr pre Radiation	0.199	2.78

Key Research Accomplishments

Selenite inhibited cell growth and induced apoptosis in androgen-dependent LAPC-4 human prostate cancer cells *in vitro*

Primary cultures of normal prostate epithelial cells were more resistant to selenite-induced apoptosis than LAPC-4 cells

Selenite-induced apoptosis in LAPC-4 cells was associated with decreased GSH:GSSG and bcl-2:bax ratios

Selenite inhibited the growth of androgen-dependent and androgen-independent LAPC-4 xenograft tumors in nude mice without systemic toxicity

Using patient-matched pairs of normal and malignant prostate cells we have shown that prostate cancer-derived cells are more sensitive to selenite-induced apoptosis than the corresponding normal cells

Intracellular GSH levels were similar in normal and cancer cells derived from the same patient, therefore, altered GSH content does not appear to mediate their differential response to selenite

Normal cells had increased MnSOD expression and SOD activity compared to cancer cells

Increasing MnSOD activity in cancer cells protected against selenite-induced apoptosis

Increased MnSOD expression in normal cells may be a predictive marker for the therapeutic response to selenite

LAPC-4 and DU 145 cells pretreated with selenite showed increased sensitivity to γ-irradiation

Reportable Outcomes

Husbeck, B, Peehl, DM, Knox, SJ. Redox modulation of human prostate carcinoma cells by selenite increases radiation-induced cell killing. Free Rad Biol Med. 38: 50-57, 2005.

Stanford Patent Application (S03-309) Methods for Treating A Neoplastic Disease In a Subject Using Inorganic Selenium-Containing Compounds

Conclusions

We have characterized the response of LAPC-4 prostate cancer cells and patient-matched pairs of normal and malignant prostate cells to selenite *in vitro*. Selenite-induced apoptosis was found to correlate with changes in Bcl-2 family member expression and altered intracellular GSH status. Normal prostate cells were more resistant to selenite-induced apoptosis than cancer cells, and this differential response may be due to altered MnSOD expression. Selenite was also found to enhance the response of prostate cancer cells to γ-irradiation. We believe that selenite has great potential as a radiosensitizer because it consumes GSH, generates free radicals, and shows selective tumor killing in prostate cancer. Importantly, both androgen-dependent and androgen-independent prostate cancer cells were sensitized to ionizing radiation by selenite. The majority of deaths from prostate cancer are due to the progression to hormone refractory disease, which is often poorly responsive to conventional therapy. Therefore, these findings could have broad clinical significance for the treatment of patients with prostate cancer and suggest that combined therapy using selenite and radiation could potentially increase local control and cure rates for patients with localized prostate cancer. Although these results show that high levels of selenite can induce apoptosis and sensitize prostate cancer cells to radiation, it is not clear whether these findings can be extrapolated to animal models or humans. Results from our limited studies, which have shown that high levels of selenite can retard tumor growth *in vivo* without apparent ill effects on the host, suggest that the use of selenite alone and in combination with radiation for the treatment of tumors is worthy of further study. However many questions remain regarding the types of tumors that may be sensitive to selenite and the dosage regimens that are safe and effective. The results of these future studies may have near-term translational potential and provide proof of principle that selenite can be used to treat prostate cancer.

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